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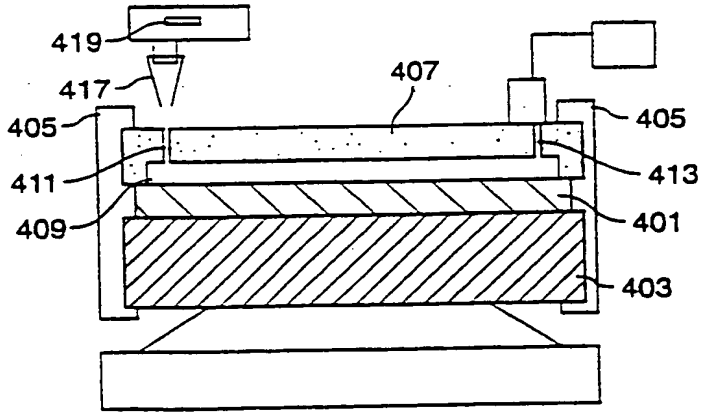


(57) Abstract

A method and device for forming large arrays of polymers on a substrate (401). According to a preferred aspect of the invention, the substrate is contacted by a channel block (407) having channels (409) therein. Selected reagents are delivered through the channels, the substrate is rotated by a rotating stage (403), and the process is repeated to form arrays of polymers on the substrate. The method may be combined with light-directed methodologies.

COMBINATORIAL STRATEGIES FOR POLYMER SYNTHESIS

BACKGROUND OF THE INVENTION

This application is related to U.S. Serial No. 796,243 (filed November 22, 1991) and to U.S. Serial No. 874,849 (filed April 24, 1992), both of which are incorporated herein by reference for all purposes.

The present invention relates to the field of polymer synthesis and screening. More specifically, in one embodiment the invention provides an improved method and system for synthesizing arrays of diverse polymer sequences. According to a specific aspect of the invention, a method of synthesizing diverse polymer sequences such as peptides or oligonucleotides is provided. The diverse polymer sequences may be used, for example, in screening studies for determination of binding affinity.

Methods of synthesizing desired polymer sequences such as peptide sequences are well known to those of skill in the art. Methods of synthesizing oligonucleotides are found in, for example, Oligonucleotide Synthesis: A Practical Approach, Gate, ed., IRL Press, Oxford (1984), incorporated herein by reference in its entirety for all purposes. The so-called "Merrifield" solid phase peptide synthesis has been in common use for several years and is described in Merrifield, J. Am. Chem. Soc. (1963) 85:2149-2154, incorporated herein by reference for all purposes. Solid-phase synthesis techniques have been provided for the synthesis of several peptide sequences on, for example, a number of "pins." See e.g., Geysen et al., J. Immun. Meth. (1987) 102:259-274, incorporated herein by reference for all purposes. Other solid-phase techniques involve, for example, synthesis of various peptide sequences on different cellulose disks supported in a column. See Frank and Doring, Tetrahedron (1988) 44:6031-6040, incorporated herein by reference for all purposes. Still other solid-phase techniques are described in U.S. Patent No. 4,728,502 issued to Hamill and WO 90/00626 (Beattie, inventor).

Each of the above techniques produces only a relatively low density array of polymers. For example, the technique described in Geysen et al. is limited to producing 96 different polymers on pins spaced in the dimensions of a standard microtiter plate.

Improved methods of forming large arrays of peptides, oligonucleotides, and other polymer sequences in a short period of time have been devised. Of particular note, Pirrung et al., U.S. Patent No. 5,143,854 (see also PCT Application No. WO 90/15070) and Fodor et al., PCT Publication No. WO 92/10092, all incorporated herein by reference, disclose methods of forming vast arrays of peptides and other polymer sequences using, for example, light-

the channels directly, filling the channels and "striping" the substrate with a first reagent, coupling a first group of monomers thereto. The first group of monomers need not be homogenous. For example, a monomer A may be placed in a first group of the channels, a monomer B in a second group of channels, and a monomer C in a third group of channels. The channels may in some embodiments thereafter be provided with additional reagents, providing coupling of additional monomers to the first group of monomers. The block is then translated or rotated, again placed on the substrate, and the process is repeated with a second reagent, coupling a second group of monomers to different regions of the substrate. The process is repeated until a diverse set of polymers of desired sequence and length is formed on the substrate. By virtue of the process, a number of polymers having diverse monomer sequences such as peptides or oligonucleotides are formed on the substrate at known locations.

According to the second aspect of the invention, a series of microchannels or microgrooves are formed on a substrate, along with an appropriate array of microvalves. The channels and valves are used to flow selected reagents over a derivatized surface. The microvalves are used to determine which of the channels are opened for any particular coupling step.

Accordingly, one embodiment of the invention provides a method of forming diverse polymer sequences on a single substrate, the substrate comprising a surface with a plurality of selected regions. The method includes the steps of forming a plurality of channels adjacent the surface, the channels at least partially having a wall thereof defined by a portion of the selected regions; and placing selected reagents in the channels to synthesize polymer sequences at the portion of the selected regions, the portion of the selected regions comprising polymers with a sequence of monomers different from polymers in at least one other of the selected regions. In alternative embodiments, the channels or flow paths themselves constitute the selected reaction regions. For example, the substrate may be a series of adjoining parallel channels, each having reaction sites therein.

According to a third aspect of the invention, a substrate is provided which has an array of discrete reaction regions separated from one another by inert regions. In one embodiment, a first monomer solution is spotted on a first set of reaction regions of a suitably derivatized substrate. Thereafter, a second monomer solution is spotted on a second set of regions, a third monomer solution is spotted on a third set and so on, until a number of the regions each have one species of monomer located therein. These monomers are reacted with the surface, and the substrate is subsequently washed and prepared for reaction with a new set of

Fig. 10 is a diagram of a flow system used to deliver coupling compounds and reagents to a flow cell;

Figs. 11a and 11b show an apparatus used to transfer a substrate from one channel block to another;

Fig. 12 is a diagram of a multichannel solid-phase synthesizer;

Figs. 13a and 13b illustrate alternative arrangements of the grooves in a channel block;

Fig. 14 is a schematic illustration of reaction pathways used to prepare some hydrophobic groups of the present invention;

Figs. 15a and 15b illustrate a microvalve device;

Figs. 16a and 16b illustrate an alternative embodiment of the invention;

Fig. 17 is a mapping of expected fluorescent intensities with a substrate selectively exposed to fluorescent dye.

steps in the synthesis of a polymer. Furthermore, each of the sets may include protected members which are modified after synthesis. The invention is described herein primarily with regard to the preparation of molecules containing sequences of monomers such as amino acids, but could readily be applied in the preparation of other polymers. Such polymers include, for example, both linear and cyclic polymers of nucleic acids, polysaccharides, phospholipids, and peptides having either α-, β-, or ω-amino acids, heteropolymers in which a known drug is covalently bound to any of the above, polynucleotides, polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes, polyimides, polyacetates, or other polymers which will be apparent upon review of this disclosure. Such polymers are "diverse" when polymers having different monomer sequences are formed at different predefined regions of a substrate. Methods of cyclization and polymer reversal of polymers are disclosed in copending application Serial No. 796,727, filed November 22, 1991, entitled "POLYMER REVERSAL ON SOLID SURFACES," incorporated herein by reference for all purposes.

3. Peptide: A peptide is a polymer in which the monomers are alpha amino acids and are joined together through amide bonds, alternatively referred to as a polypeptide. Amino acids may be the L-optical isomer or the D-optical isomer. Peptides are two or more amino acid monomers long and are often more than 20 amino acid monomers long. Standard abbreviations for amino acids are used (e.g., P for proline). These abbreviations are included in Stryer, Biochemistry, Third Ed., 1988, which is incorporated herein by reference for all purposes.

4. Receptor: A receptor is a molecule that has an affinity for a ligand. Receptors may be naturally-occurring or manmade molecules. They can be employed in their unaltered state or as aggregates with other species. Receptors may be attached, covalently or noncovalently, to a binding member, either directly or via a specific binding substance. Examples of receptors which can be employed by this invention include, but are not restricted to, antibodies, cell membrane receptors, monoclonal antibodies and antisera reactive with specific antigenic determinants, viruses, cells, drugs, polynucleotides, nucleic acids, peptides, cofactors, lectins, sugars, polysaccharides, cellular membranes, and organelles. Receptors are sometimes referred to in the art as anti-ligands. As the

WO 90/05749, and WO 90/05785, which are incorporated herein by reference for all purposes.

f) Hormone receptors: Determination of the ligands which bind with high affinity to a receptor such as the receptors for insulin and growth hormone is useful in the development of, for example, an oral replacement of the daily injections which diabetics must take to relieve the symptoms of diabetes or a replacement for growth hormone. Other examples of hormone receptors include the vasoconstrictive hormone receptors; determination of ligands for these receptors may lead to the development of drugs to control blood pressure.

g) Opiate receptors: Determination of ligands which bind to the opiate receptors in the brain is useful in the development of less-addictive replacements for morphine and related drugs.

5. Substrate: A material having a rigid or semi-rigid surface. In many embodiments, at least one surface of the substrate will be substantially flat, although in some embodiments it may be desirable to physically separate synthesis regions for different polymers with, for example, wells, raised regions, etched trenches, or the like. In some embodiments, the substrate itself contains wells, trenches, flow through regions, etc. which form all or part of the synthesis regions. According to other embodiments, small beads may be provided on the surface, and compounds synthesized thereon may be released upon completion of the synthesis.

6. Channel Block: A material having a plurality of grooves or recessed regions on a surface thereof. The grooves or recessed regions may take on a variety of geometric configurations, including but not limited to stripes, circles, serpentine paths, or the like. Channel blocks may be prepared in a variety of manners, including etching silicon blocks, molding or pressing polymers, etc.

7. Protecting Group: A material which is bound to a monomer unit and which may be selectively removed therefrom to expose an active site such as, in the specific example of an amino acid, an amine group. Specific examples of photolabile protecting groups are discussed in Fodor et al., PCT Publication No. WO 92/10092 (previously incorporated by reference) and U.S. Serial No. _____ filed November 2, 1992 (attorney docket No. 11509-68) incorporated herein by reference for all purposes.

oligonucleotides in selected regions of a substrate. Such substrates having the diverse sequences formed thereon may be used in, for example, screening studies to evaluate their interaction with receptors such as antibodies and nucleic acids. For example, in preferred embodiments the invention provides for screening of peptides to determine which if any of a diverse set of peptides has a strong binding affinity with a receptor and, in most preferred embodiments, to determine the relative binding affinity of various peptides with a receptor of interest.

Such diverse polymer sequences are preferably synthesized on a single substrate. By synthesizing the diverse polymer sequences on a single substrate, processing of the sequences to evaluate characteristics such as relative binding affinity is more easily conducted. By way of example, when an array of peptide sequences (or a library of other compounds) is to be evaluated to determine the peptides' relative binding affinity to a receptor, the entire substrate and, therefore, all or a group of the polymer sequences may be exposed to an appropriately labelled receptor and evaluated simultaneously.

In some embodiments, the present invention can be employed to localize and, in some cases, immobilize vast collections of synthetic chemical compounds or natural product extracts. In such methods, compounds are deposited on predefined regions of a substrate. The reaction of the immobilized compound (or compounds) with various test compositions such as the members of the chemical library or a biological extract are tested by dispensing small aliquots of each member of the library or extract to a different region. Competitive assays or other well-known techniques can be used to identify a desired activity. As an example, a large collection of human receptors is deposited on a substrate, one in each region to form an array. A plant/animal extract is then screened for binding to various receptors of the array.

The present invention has certain features in common with the "light directed" methods described in U.S. Patent No. 5,143,854, previously incorporated by reference. The light directed methods discussed in the '854 patent involve activating predefined regions of the substrate and then contacting the substrate with a preselected monomer solution. The predefined regions can be activated with a light source shown through a mask (much in the manner of photolithography techniques used in integrated circuit fabrication). Other regions of the substrate remain inactive because they are blocked by the mask from illumination. Thus, a light pattern defines which regions of the substrate react with a given monomer. By repeatedly activating different sets of predefined regions and contacting different monomer solutions with the substrate, a diverse

block on the surface of the substrate, a reagent having the monomer A flows through or is placed in all or some of the channel(s). The channels provide fluid contact to the first selected regions, thereby binding the monomer A on the substrate directly or indirectly (via a linker) in the first selected regions.

Thereafter, a monomer B is coupled to second selected regions, some of which may be included among the first selected regions. The second selected regions will be in fluid contact with a second flow channel(s) through translation, rotation, or replacement of the channel block on the surface of the substrate; through opening or closing a selected valve; or through deposition of a layer of photoresist. If necessary, a step is performed for activating at least the second regions. Thereafter, the monomer B is flowed through or placed in the second flow channel(s), binding monomer B at the second selected locations. In this particular example, the resulting sequences bound to the substrate at this stage of processing will be, for example, A, B, and AB. The process is repeated to form a vast array of sequences of desired length at known locations on the substrate.

After the substrate is activated, monomer A can be flowed through some of the channels, monomer B can be flowed through other channels, a monomer C can be flowed through still other channels, etc. In this manner, many or all of the reaction regions are reacted with a monomer before the channel block must be moved or the substrate must be washed and/or reactivated. By making use of many or all of the available reaction regions simultaneously, the number of washing and activation steps can be minimized.

Various embodiments of the invention will provide for alternative methods of forming channels or otherwise protecting a portion of the surface of the substrate. For example, according to some embodiments, a protective coating such as a hydrophilic or hydrophobic coating (depending upon the nature of the solvent) is utilized over portions of the substrate to be protected, sometimes in combination with materials that facilitate wetting by the reactant solution in other regions. In this manner, the flowing solutions are further prevented from passing outside of their designated flow paths.

The "spotting" embodiments of the present invention can be implemented in much the same manner as the flow channel embodiments. For example, a monomer A can be delivered to and coupled with a first group of reaction regions which have been appropriately activated. Thereafter, a monomer B can be delivered to and reacted with a second group of activated reaction regions. Unlike the flow channel embodiments described above, reactants are delivered by directly depositing (rather than flowing) relatively

length of the substrate adjacent to the x_1 and x_2 channels. Each coupling step may in some embodiments be composed of a variety of substeps. For example, each coupling step may include one or more substeps for washing, chemical activation, or the like.

Thereafter or concurrently therewith, as shown in Fig. 2, a second monomer B is provided to selected flow channels and the monomer B binds to the substrate where the second flow channels provide contact therewith. In the particular example shown in Fig. 2, monomer B is bound along channels x_3 and x_4. When the monomers A and B flow through their respective flow channels simultaneously, only a single process step is required to perform two coupling steps simultaneously. As used herein, a "process step" refers to the injection of one or more channels with one or more reagents. A "coupling step" refers to the addition of a monomer in a polymer.

Processing thereafter continues in a similar manner with monomers C and D in the manner shown in the flow diagram of Fig. 2, with monomer C being bound in the flow channels y_1 and y_2, and D being bound in the flow channels y_3 and y_4. Preferably, monomers C and D are directed through the flow channels y_1 to y_4 simultaneously whereby two coupling steps are performed with a single process step. Light regions in Fig. 1 indicate the intersections of the resulting flow paths.

Fig. 3 illustrates the mapping of sequences formed using the above illustrated steps. As shown therein, the sequences A, B, C, D, AD, BD, AC, and BC have been formed using only two process steps. Accordingly, it is seen that the process provides for the synthesis of vast arrays of polymer sequences using only a relatively few process steps. By way of further example, it is necessary to use only two process steps to form all of the $4^2 = 16$ dimers of a four-monomer basis set. By way of further example, to form all 4^8 octomers of a four-monomer basis set, it is necessary to provide only 256 flow channels oriented in the "x" direction, and 256 flow channels oriented in the "y" direction, with a total of eight coupling steps.

The power of the technique is further illustrated by synthesizing the complete array of six hexamer peptides from a 20 amino acid basis set. This array will include 20^6 or 64,000,000 regions defining 64,000,000 different peptides and can be formed in only six process steps. Further, the method requires only three different templates, one having 20 parallel channels, a second having 400 channels each 1/20th as wide as the first, and a third having 8000 channels each 1/20th as wide as the second. Each template will be used in two process steps, each at an orientation at 90 degrees with respect to the other as illustrated in Fig. 4. With the first

admitted through gas pressure inlet 103 to provide clamping pressure to immobilize the substrate while fluids are flowed from fluid flow inlet 115, through channel 123, and out fluid outlet 117. The upper and lower portions of the pressure chamber housing 105 and 125 are held together by nuts 121 and bolts 104. Of course, other means such as clamps can be used to hold the pressure chamber housing portions together.

Fig. 7 illustrates preferred flow path configurations in channel blocks of the present invention. As shown in Figs. 7a, fluid delivery sites 127, 129, 131, 133, 135, and 137 are connected to channels leading to reaction region 141. A similar arrangement is shown for comparison in Fig. 7b where the orientation of the flow channels in the reaction regions is shifted by 90 degrees on a rectangular channel block. Vacuum ports 145 and 146 to an external vacuum line are provided so that substrate position is maintained during fluid flow.

The channels shown in Figs. 7a and 7b form a "fanned channel array" on channel block 139 in a manner analogous to that of the lead pattern employed in integrated circuits. This provides significantly increased separation of fluid delivery points in comparison to the high density of channels in the reaction region. In a 2 inch by 3 inch substrate, at least about a 4:1 increase in spatial separation typically can be attained by the fanned arrangement. Thus, if the channels in the reaction regions are separated by 200 microns, the delivery ports can be separated by 0.8 mm.

The spatial separation can be further increased by staggering the delivery ports as shown for ports 127, 129, and 131. This can provide an additional channel separation of at least about 3:1. Thus, for the channels separated by 200 microns, a staggered fanned array provides 2.4 mm separation between the delivery ports. Thus, fluid can be delivered to a high-density array of channels in the reaction region from standard 1.6 mm Teflon™ tubing. If additional spacing is necessary, the substrate size can be increased, while preserving the reaction region size.

As shown in Fig. 8, the fluid delivery ports are accessed from holes in the back surface of a stabilizing plate 108 on the channel block. The stabilizing plate, which is preferably made from fused pyrex, provides structural integrity to the channel block during clamping in the pressure chamber. It may also provide a means to access the channel block ports and reduce leakage between ports or channels. In preferred embodiments, the channels 123 of the channel block are formed on a wafer 106 which generally may be any machinable or cast material, and preferably may be etched silicon or a micromachined ceramic. In other embodiments, the channel block is

steps during a chemical synthesis. One block forms a horizontal array on the solid substrate, while the other block forms a vertical array. To create a matrix of intersecting rows and columns of chemical compounds, the solid substrate is transferred from one block to the other during successive process steps. While many experiments require only a single transfer from one block to the other during a series of process steps, the fanned channel array transfer block 75 illustrated in Figs. 11a and 11b provides one device for maintaining accurate registration of the solid substrate 71 relative to the channel blocks 79 during repeated transfers. In some embodiments, a single channel block can be used for horizontal and vertical arrays by simply rotating it by 90 degrees as necessary.

The transfer block is positioned with respect to the channel block so that the dimensional characteristics of the solid substrate are not used in the alignment. The transfer block 75 is aligned to the channel block by kinematic mount 81 while vacuum is switched from vacuum line 83 on the channel block to vacuum line 77 on the transfer block (during normal operation, a vacuum holds the substrate against the channel block). The substrate and transfer block are then moved and repositioned relative to the second channel bock. Vacuum is then switched to the second channel block, retaining the substrate in proper alignment. This way, accurate registration can be assured between process steps regardless of variation in the dimensions of individual substrates. The transfer block system also maintains alignment of the matrix area during transfers to and from the flow cell during experiments utilizing both mechanical and light-directed process steps.

In some embodiments the channel block need not be utilized. Instead, in some embodiments, small "strips" of reagent are applied to the substrate by, for example, striping the substrate or channels therein with a pipettor. Such embodiments bear some resemblance to the spotting embodiments of this invention. According to other embodiments the channels will be formed by depositing a photoresist such as those used extensively in the semiconductor industry. Such materials include polymethyl methacrylate (PMMA) and its derivatives, and electron beam resists such as poly(olefin sulfones) and the like (more fully described in Ghandi, "VLSI Fabrication Principles," Wiley (1983) Chapter 10, incorporated herein by reference in its entirety for all purposes). According to these embodiments, a resist is deposited, selectively exposed, and etched, leaving a portion of the substrate exposed for coupling. These steps of depositing resist, selectively removing resist and monomer coupling are repeated to form polymers of desired sequence at desired locations.

solvents, methylene chloride, DMF, ethyl alcohol, or the like. Optionally, the substrate may be provided with appropriate linker molecules on the surface thereof. The linker molecules may be, for example, aryl acetylene, ethylene glycol oligomers containing from 2-10 monomers or more, diamines, diacids, amino acids, or combinations thereof. Thereafter, the surface is provided with protected surface active groups such as TBOC or FMOC protected amino acids. Such techniques are well known to those of skill in the art.

Thereafter, the channel block and the substrate are brought into contact forming fluid-tight channels bounded by the grooves in the channel block and the substrate. When the channel block and the substrate are in contact, a protecting group removal agent is, thereafter, directed through a first selected channel or group of channels by placing the pipettor on the flow inlet of the selected channel and, optionally, the vacuum source on the outlet of the channel. In the case of, for example, TBOC protected amino acids, this protecting group removal agent may be, for example, trifluoroacetic acid (TFA). This step is optionally followed by steps of washing to remove excess TFA with, for example, dichloromethane (DCM).

Thereafter, a first amino acid or other monomer A is directed through the first selected flow channel. Preferably this first amino acid is also provided with an appropriate protecting group such as TBOC, FMOC, NVOC, or the like. This step is also followed by appropriate washing steps. The of deprotection/coupling steps employed in the first group of channels are concurrently with or thereafter repeated in additional groups of channels. In preferred embodiments, monomer A will be directed through the first group of channels, monomer B will be directed through a second group of flow channels, etc., so that a variety of different monomers are coupled on parallel channels of the substrate.

Thereafter, the substrate and the channel block are separated and, optionally, the entire substrate is washed with an appropriate material to remove any unwanted materials from the points where the channels contact the substrate.

The substrate and/or block is then, optionally, washed and translated and/or rotated with the stage. In preferred embodiments, the substrate is rotated 90 degrees from its original position, although some embodiments may provide for greater or less rotation, such as from 0 to 180 degrees. In other embodiments, such as those discussed in connection with the device shown in Fig. 7, two or more different channel blocks are employed to produce different flow patterns across the substrate. When the channel block is rotated, it may simultaneously be translated with respect to the substrate. "Translated" means any relative motion of the substrate

filled with resin or reaction mixture. The gasketing allows close contact between the support matrix 1002 and a "mask" (not shown). The mask serves to control delivery of a first group reactant solutions through predetermined lines (tubes) to a first set of reaction regions. By ensuring close contact between the delivery tubes 1000, the mask, and the support matrix 1002, the probability that reaction solutions will be accidently added to the wrong reaction site is reduced.

After each process step, the mask can be changed so that a new group reactants is delivered to a new set of reaction regions. In this manner, a combinatorial strategy can be employed to prepare a large array of polymers or other compounds. In other embodiments, mechanisms other than masks can be employed to block the individual delivery tubes. For example, an array of control valves within the tubes may be suitable for some embodiments.

By adjusting the thickness of the synthesis support matrix, the quantity of immobilized material in the reaction regions can be controlled. For example, relatively thin support synthesis matrices can be used to produce small amounts of surface bound oligomers for analysis, while thicker support matrices can be used to synthesize relatively large quantities of oligomers which can be cleaved from the support for further use. In the latter embodiment, a collector having dimensions matching the individual synthesis supports can be employed to collect oligomers that are ultimately freed from the reaction matrix.

To illustrate the ability of this system to synthesize numerous polymers, a square synthesis matrix measuring 10 cm along each side and having 5 mm reaction regions separated by 5 mm wide gaskets provides 100 individual syntheses sites (reaction regions). By reducing the size of the reaction regions to 2.5 mm on each side, 400 reactions regions become available.

While linear grooves are shown herein in the preferred aspects of the invention, other embodiments of the invention will provide for circular rings or other shapes such as circular rings with radial grooves running between selected rings. According to some embodiments, channel blocks with different geometric configurations will be used from one step to the next, such as circular rings in one step and linear stripes in the next. Fig. 13a illustrates one of the possible arrangements in which the channels 409 are arranged in a serpentine arrangement in the channel block 407. Through appropriate translation and/or rotation of the channel block, polymers of desired monomer sequence are formed at the intersection of the channels during successive polymer additions, such as at location 501, where the intersection of a previous or subsequent set of channels is shown in dashed lines. Fig. 13b

locations of the antibody of interest. For example, in one specific embodiment, if a mouse antibody is to be studied, a labelled second antibody may be exposed to the substrate which is, for example, goat antimouse. Such techniques are described in PCT Publication No. WO92/10092, previously incorporated herein by reference.

V. Spotting Embodiments

According to some embodiments, monomers (or other reactants) are deposited from a dispenser in droplets that fill predefined regions. For example, in a single coupling step, the dispenser deposits a first monomer in a series of predefined regions by moving over a first region, dispensing a droplet, moving to a second region, dispensing a droplet, and so on until the each of the selected regions has received the monomer. Next the dispenser deposits a second monomer in a second series of predefined regions in much the same manner. In some embodiments, more than one dispenser may be used so that more than one monomer are simultaneously deposited. The monomers may react immediately on contact with the reaction regions or may require a further activation step, such as the addition of catalyst. After some number of monomers have been deposited and reacted in predefined regions throughout the substrate, the unreacted monomer solution is removed from the substrate. This completes a first process step.

For purposes of this embodiment, the spacing between the individual reaction regions of the substrate preferably will be less than about 3 mm, and more preferably between about 5 and 100 μm. Further, the angular relation between the regions is preferably consistent to within 1 degree and more preferably to within 0.1 degree. Preferably, the substrate will include at least about 100 reaction regions, more preferably at least about 1000 reaction regions, and most preferably at least about 10,000 reaction regions. Of course, the density of reaction regions on the substrate will vary. In preferred embodiments, there are at least about 1000 reaction regions per cm^2 of substrate, and more preferably at least about 10,000 regions per cm^2.

To deposit reactant droplets consistently at precisely specified regions, a frame of reference common to the delivery instrument and the substrate is required. In other words, the reference coordinates of the instrument must be accurately mapped onto the reference coordinates of the substrate. Ideally, only two reference points on the substrate are necessary to map the array of polymer regions completely. The dispenser instrument locates these reference points and then adjusts its internal reference coordinates to provide the necessary mapping. After this, the dispenser can move a particular distance in a particular direction and be positioned

deposits correctly metered amounts of monomer. Analogous systems widely used in the microelectronic device fabrication and testing arts can move at rates of up to 3-10 stops per second. The translational (X-Y) accuracy of such systems is well within 1 μm.

Translational mechanisms for moving the dispenser are preferably equipped with closed loop position feedback mechanisms (encoders) and have insignificant backlash and hysteresis. In preferred embodiments, the translation mechanism has a high resolution, i.e. better than one motor tick per encoder count. Further, the electro-mechanical mechanism preferably has a high repeatability relative to the reaction region diameter travel distance (typically ± 1μm or better).

To deposit a drop of monomer solution on the substrate accurately, the dispenser nozzle must be placed a correct distance above the surface. In one embodiment, the dispenser tip preferably will be located about 5-50 μm above the substrate surface when a five nanoliter drop is released. More preferably, the drop will be about 10 μm above the substrate surface when the drop is released. The degree of control necessary to achieve such accuracy is attained with a repeatable high-resolution translation mechanism of the type described above. In one embodiment, the height above the substrate is determined by moving the dispenser toward the substrate in small increments, until the dispenser tip touches the substrate. At this point, the dispenser is moved away from the surface a fixed number of increments which corresponds to a specific distance. From there the drop is released to the cell below. Preferably, the increments in which the dispenser moves less than about 5 μm and more preferably less than about 2 μm.

In an alternative embodiment, the dispenser nozzle is encircled by a sheath that rigidly extends a fixed distance beyond the dispenser tip. Preferably, this distance corresponds to the distance the solution drop will fall when delivered to the selected reaction region. Thus, when the sheath contacts the substrate surface, the movement of the dispenser is halted and the drop is released. It is not necessary in this embodiment to move the dispenser back, away from the substrate, after contact is made. The point of contact with the surface can be determined by a variety of techniques such as by monitoring the capacitance or resistance between the tip of the dispenser (or sheath) and the substrate below. A rapid change in either of these properties is observed upon contact with the surface.

To this point, the spotting system has been described

electrophoretic pump. In this device, a thin capillary connects a reservoir of the reactant with the nozzle of the dispenser. At both ends of the capillary, electrodes are present to provide a potential difference. As is known in the art, the speed at which a chemical species travels in a potential gradient of an electrophoretic medium is governed by a variety of physical properties, including the charge density, size, and shape of the species being transported, as well as the physical and chemical properties of the transport medium itself. Under the proper conditions of potential gradient, capillary dimensions, and transport medium rheology, a hydrodynamic flow will be set up within the capillary. Thus, in an electrophoretic pump of the present invention, bulk fluid containing the reactant of interest is pumped from a reservoir to the substrate. By adjusting the appropriate position of the substrate with respect to the electrophoretic pump nozzle, the reactant solution is precisely delivered to predefined reaction regions.

In one particularly useful application, the electrophoretic pump is used to produce an array containing various fractions of an unknown reactant solution. For example, an extract from a biological material such as leaf or a cell culture might contain various unknown materials, including receptors, ligands, alkaloids, nucleic acids, and even biological cells, some of which may have a desired activity. If a reservoir of such extract is electrophoretically pumped, the various species contained therein will move through the capillary at different rates. Of course, the various components being pumped should have some charge so that they can be separated. If the substrate is moved with respect to the dispenser while the extract components are being separated electrophoretically, an array containing various independent species is produced. This array is then tested for activity in a binding assay or other appropriate test. Those elements of the array that show promising activity are correlated with a fraction of the extract which is subsequently isolated from another source for further study. In some embodiments, the components in the extract solution are tagged with, for example, a fluorescent label. Then, during the process of delivering the solution with the electrophoretic pump, a fluorescence detector determines when labeled species are being deposited on the substrate. In some embodiments, the tag selectively binds to certain types of compounds within the extract, and imparts a charge to those compounds.

Other suitable delivery means include osmotic pumps and cell (biological) sorters. An osmotic pump delivers a steady flow of solution for a relatively long period. The construction of such pumps is well-known in the art, generally incorporating a solution of the extract of interest within a solvent permeable bag. Osmotic

chemicals is easily measured by a variety of techniques such as those described in Adamson, Physical Chemistry of Surfaces, John Wiley and Sons, 5th Ed. (1990) which is incorporated herein by reference for all purposes. The difference of the solid-liquid and solid-air tensions can, for a given system, be determined empirically from a Zisman plot. In this approach, the contact angles are measured for a homologous series of liquids on a given solid surface. For some liquid in the series, a "critical contact angle" is observed, beyond which lower surface tension liquids wet the surface. The liquid-air surface tension of the liquid at this critical contact angle is assumed to be the surface tension of the solid. This approach has been found to provide quite reasonable results for low energy solids such as Teflon, polyethylene, hydrocarbons, etc. The information gained from such studies is used to optimize substrate compositions to increase wetting angles for given reactant solutions in the array.

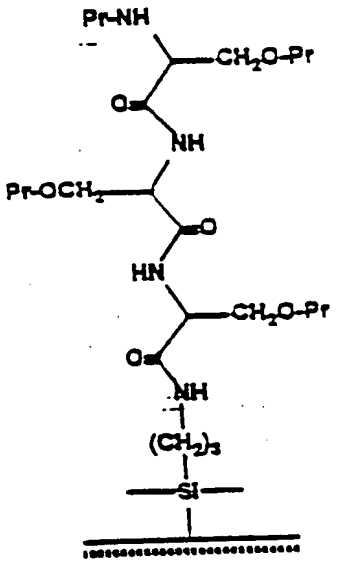
Methods for controlling chemical composition and therefore the local surface free energy of a substrate surface include a variety of techniques apparent to those skilled in the art. Chemical vapor deposition and other techniques applied in the fabrication of integrated circuits can be applied to deposit highly uniform layers on selected regions of a surface. As a specific example, the wettability of a silicon wafer surface has been manipulated on the micrometer scale through a combination of self-assembled monolayer depositions and micromachining. See Abbott et al., "Manipulation of the Wettability of Surfaces on the .1 to 1 Micrometer Scale Through Micromachining and Molecular Self-Assembly" Science, 257 (Sept. 4, 1992) which is incorporated herein by reference for all purposes.

In a preferred embodiment, the perimeters of the individual regions are formed on a hydrophilic substrate defined by selectively removing hydrophobic protecting groups from the substrate surface. For example, a mono-layer of hydrophobic photoprotecting groups can be coupled to, for example, linker molecules attached to the substrate surface. The surface then is selectively irradiated (or otherwise activated by, for example, acid) through a mask to expose those areas where the reaction regions are to be located. This cleaves the protecting groups from the substrate surface, causing the reaction regions to be less hydrophobic than the surrounding area. This process produces a high density of reaction regions on the substrate surface. Because hydrophobic materials have lower surface free energies (surface tensions) than water, the solution droplet in the cell beads rather than spreads.

In some preferred embodiments, the substrate is prepared by first covalently attaching a monolayer of the desired reactive functional group (e.g. amine, hydroxyl, carboxyl, thio, etc.), which

hydrophobicity to glass surfaces to which a member of the class is attached. The hydrophobicity of the basic nitrobenzyl protecting group is enhanced by appending group chain hydrocarbon substituent. Exemplary hydrophobic chains include $C_{12}H_{25}$ (lauryl) or $C_{18}H_{37}$ (stearyl) substituents. The syntheses of suitably activated forms (bromide, chloromethyl ether, and oxycarbonyl chloride) of a typical protecting group is schematically outlined in Fig. 14.

The spacer group ("Y") contributes to the net hydrophobic or hydrophilic nature of the surface. For example, those spacers consisting primarily of hydrocarbon chains, such as $-(CH_2)_a-$, tend to decrease wettability. Spacers including polyoxyethylene ($-(CH_2CH_2O)_b-$), or polyamide ($-(CH_2CONH)_c-$) chains tend to make the surface more hydrophilic. An even greater effect is achieved by using spacer groups which possess, in addition to the protected functional group, several "masked" hydrophilic moieties. This is illustrated below.



In preferred embodiments, the hydrophilic reaction regions is a two-dimensional circle or other shape having an aspect ratio near one (i.e. the length is not substantially larger or smaller than the width). However, in other embodiments, the hydrophilic region may take the form of a long channel which is used to direct flowing reactants in the manner described above.

The arrays produced according to the above spotting embodiments are generally used in much the same manner as the arrays produced by the flow channel embodiments described above. For example, the arrays can be used in screening with fluorescein labelled receptors as described in PCT Publication No. WO92/10092, previously incorporated by reference.

VI. Alternative Embodiments

According to some embodiments of the invention, microvalve structures are used to form channels along selected flow paths on the substrate. According to these embodiments, an array of microvalves is formed and operated by an overlying or underlying array of electrodes that is used to energize selected valves to open and close such valves.

Fig. 15 illustrates such a structure, Fig. 15a illustrating the system in end view cross-section and Fig. 15b illustrating the system in top view. The structure shown therein provides for only two synthesis chambers for the purpose of clarity, but in most embodiments a far greater number of chambers will be provided. Microvalves are discussed in detail in, for example, Zdeblick, U.S. Patent No. 4,966,646, and Knutti, "Advanced Silicon Microstructures," ASICT Conference (1989), both incorporated herein by reference for all purposes.

As shown therein, a substrate 602 is provided with a plurality of channels 604 formed using photolithographic, or other related techniques. The channels lead up to a synthesis chamber 606. At the end of each channel is valve structure 608. As shown in Fig. 15, the channels lead up to the chambers, but may be isolated from the chambers by the valves. Multiple valves may be provided for each chamber. In the particular structure shown in Fig. 15, the right valve on the left chamber and the left valve on the right chamber are open while the remaining valves are closed. Accordingly, if reagent is delivered to the top of the substrate, it will flow through the open channel to and through the chamber on the left, but not the one on the right. Accordingly, coupling steps may be conducted on the chamber with selected reagents directed to selected chambers, using the techniques discussed above.

According to some embodiments, a valve is supplied on one side of the chamber 606, but the valve on the opposite side is replaced by a semi-permeable membrane. According to these embodiments, it becomes possible to flow a selected reagent into the chamber 606 and, thereafter, flow another selected reagent through the flow channel adjacent the semi-permeable membrane. The semi-permeable membrane will permit a portion of the material on one side

channel width, to provide coupling of a monomer in the regions between the original channels.

Thereafter, the process of directed irradiation by light, followed by coupling with the channel block is repeated at the previously unexposed regions. The process is then preferably repeated again, with the stripes of the mask and the channel block rotated at, for example, 90 degrees. The coupling steps will provide for the formation of polymers having diverse monomer sequences at selected regions of the substrate through appropriate translation of the mask and substrate, and through appropriate mask selection. Through a combination of the light-directed techniques and the mechanical flow channel techniques disclosed herein, greater efficiency in forming diverse sequences is achieved, because multiple monomers are coupled in a single irradiation/coupling step.

In light-directed methods, the light shown through the mask is diffracted to varying degrees around the edges of the dark regions of the mask. Thus, some undesired removal of photosensitive protecting groups at the edges of "dark" regions occurs. This effect is exacerbated by the repeated mask translations and subsequent exposures, ultimately leading to inhomogeneous synthesis sites at the edges of the predefined regions. The effect is, of course, dependent upon the thickness of the glass substrate and the angle at which the light is diffracted. If the mask is positioned on the "backside" of the substrate, a diffraction angle of 2.5° and a substrate thickness of 0.7 mm creates a 60 μm strip of light (of variable intensity) flanking each edge. For a 0.1 mm thick substrate, the strip is approximately 5 μm wide.

To reduce these "bleed-over" effects of diffraction, a pinhole mask may be employed to activate and/or define reaction regions of the substrate. Thus, for example, light shown through the pinhole mask is directed onto a substrate containing photoremovable hydrophobic groups. The groups in the illuminated regions are then removed to define hydrophilic reaction regions. In one specific embodiment, the pinhole mask contains a series of circular holes of defined diameter and separation, e.g., 20 μm diameter holes spaced 50 μm apart. In some preferred embodiments, a stationary pinhole mask is sandwiched between the substrate and a translational mask of the type described in PCT Publication No. WO92/10092. In this manner selected regions of the substrate can be activated for polymer synthesis without bleed-over. The translational mask is used to illuminate selected holes of the stationary pinhole mask, and is aligned such that its edges dissect the distance separating the holes of the stationary mask thereby eliminating diffractive removal of photoprotecting groups at neighboring sites. Because there is negligible bleed-over incident light, inhomogeneous synthesis at

contact with the substrate. The fluorescein marker was in a solution of DMF and flowed through the channels by injecting the material into the groove with a manual pipet.

Fluorescein dye was similarly injected into every other channel in the block, the block was rotated, and the process was repeated. The expected resulting plot of fluorescent intensity versus location is schematically illustrated in Fig. 17. Dark regions are shown at the intersections of the vertical and horizontal stripes, while lighter grey at non-intersecting regions of the stripes. The dark grey regions indicate expected regions of high dye concentration, while the light regions indicate regions of expected lower dye concentration.

A mapping was made of fluorescence intensity of a portion of an actual slide, with intensity data gathered according to the methods of PCT Publication No. WO92/10092, previously incorporated by reference. The results agree closely with the expected results, exhibiting high fluorescence intensity at the intersection of the channels (about 50% higher than non-intersecting regions of the stripes), and lower fluorescence intensity at other regions of the channels. Regions which were not exposed to fluorescence dye show little activity, indicating a good signal-to-noise ratio. Intersections have fluorescence intensity about 9x as high as background. Also, regions within the channels show low variation in fluorescence intensity, indicating that the regions are being evenly treated within the channels.

B. Formation of YGGFL

The system was used to synthesize four distinct peptides: YGGFL (SEQ. ID NO:1), YpGFL (SEQ. ID NO:2), pGGFL (SEQ. ID NO:3), and ppGFL (the abbreviations are included in Stryer, Biochemistry, Third Ed. (1988), previously incorporated herein by reference; lower case letters indicate D-optical isomers and upper case letters indicate L-optical isomers). An entire glass substrate was derivatized with TBOC-protected aminopropyltriethoxysilane, deprotected with TFA, coated with FMOC-protected caproic acid (a linker), deprotected with piperidine, and coated with FMOC-protected Glycine-Phenylalanine-Leucine (GFL).

This FMOC-GFL-coated slide was sealed to the channel block, and all 10 grooves were deprotected with piperidine in DMF. After washing the grooves, FMOC Glycine (G) was injected in the odd grooves, and FMOC d-Proline (p) was injected in the even grooves. After a two-hour coupling time, using standard coupling chemistry, all grooves were washed with DMF. The grooves were vacuum dried, the block removed and rotated 90 degrees. After resealing, all grooves were deprotected with piperidine in DMF and washed. FMOC Tyrosine

Six nucleosides were then coupled to the entire reaction region using a synthesis process consisting of deprotection, coupling, and oxidation steps for each monomer applied. These first six nucleosides were coupled in a reaction region defined by a 0.84 inch diameter circular well in an aluminum template clamped to the two by three inch slide.

The seventh and eighth monomers were applied to the substrate by flowing monomer solutions through 100 micron channels in an etched silicon channel block (employed in Example C above). The seventh base was coupled along the long axis (vertical) of the two-inch by three-inch slide, and the eighth base perpendicular to the seventh, along the short axis (horizontal) of the slide. This defined an active matrix region of 1.28 by 1.28 cm having a density of 2,500 reaction regions per square centimeter.

The channel block was centered over the reaction region and clamped to the substrate using a clamping assembly consisting of machined aluminum plates. This aligned the two inch by three inch substrate relative to the channel block in the desired orientation. Rotation of the top clamp plate and channel block relative to the bottom clamp plate and substrate between the seventh and eighth coupling steps provided for the matrix of intersecting rows and columns.

In the top clamp plate, fluid delivery wells were connected to laser-drilled holes which entered individual channels from the back surface of the channel block. These delivery wells were used to pipette coupling reagents into channels while the channel block was clamped to the substrate. Corresponding fluid-retrieval wells were connected to vacuum at the downstream of the channel block, drawing fluid through the channels and out to a waste container. Thus continuous fluid flow over the substrate in the channel region during coupling steps was achieved.

The complete octamer synthesized at the channel intersections formed by the seventh and eighth coupling steps had the following sequence:

Substrate--(3')CGCAGCCG(5') (SEQ. ID NO:4).

After completion of the synthesis process, cleavage of exocyclic amines was performed by immersion of the reaction region in concentrated ammonium hydroxide. The reaction region was then incubated at 15°C for one hour in a 10 nM solution of the complementary base sequence 5' GCGTCGGC-F (SEQ. ID No:5), where "F" is a fluorescein molecule coupled to the 3' end of the oligonucleotide. The target chain solution was then flushed from the reaction region and replaced with neat 6x SSPE buffer, also at 15°C. Finally, the reaction region was then scanned using a laser fluorescence detection system while immersed in the buffer.

SEQUENCE LISTING

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Fodor, Stephen P.A.
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Modlin, Douglas

(ii) TITLE OF INVENTION: Combinatorial Strategies For Polymer Synthesis

(iii) NUMBER OF SEQUENCES: 5

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) DOCUMENT NUMBER: US 07/796,243
(B) FILING DATE: 22-NOV-1991
(C) CLASSIFICATION:

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(A) NAME: Weaver, Jeffrey K.
(B) REGISTRATION NUMBER: 31,314
(C) REFERENCE/DOCKET NUMBER: 11509-39-1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 415-326-2600
(B) TELEFAX: 415-326-2422

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Tyr Gly Phe Leu
1

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCCGACGC

8

WHAT IS CLAIMED IS:

1. A method of forming polymers having diverse monomer sequences on a single substrate, said substrate comprising a surface with a plurality of selected regions, said method comprising the steps of:

a) forming a plurality of channels adjacent said surface, said channels at least partially having a wall thereof defined by a portion of said selected regions;

b) placing selected monomers in said channels to synthesize polymers at said portion of said selected regions, said portion of said selected regions comprising polymers with a sequence of monomers different from polymers in at least one other of said selected regions; and

c) repeating steps a) and b) with said channels formed along a second portion of said selected regions.

2. The method as recited in claim 1 wherein said step of forming a plurality of channels comprises the step of placing a channel block adjacent said surface, said channel block having a plurality of grooves therein, walls of said grooves and said surface at least partially defining said flow channels.

3. The method as recited in claim 1 wherein the step of placing selected reagents in said channels comprises the steps of:

removing a protecting group from an active site in at least a first channel;

flowing a first monomer through said at least a first channel, said first monomer comprising a protecting group thereon, said first monomer binding to said active site in said first channel;

removing a protecting group from said active site in at least a second channel, at least a portion of said second channel overlapping a portion of said substrate contacted by said first channel; and

flowing a second monomer through said at least a second channel, said second monomer binding to said active site in said second channel.

4. The method as recited in claim 1 further comprising the step of screening said polymers for binding affinity with a receptor.

5. The method as recited in claim 1 wherein at least 10 different polymers are formed on said surface.

operating said valves and flowing said selected reagents through channels formed thereby.

15. The method as recited in claim 1 preceded by the step of irradiating portions of said substrate with light whereby photoremovable groups are removed from active groups on said substrate.

16. The method as recited in claim 15 wherein said selected irradiated portions are in the form of stripes, and wherein said step of forming channels comprises forming said channels along a path of said stripes, different reagents placed in at least a portion of said channels.

17. A method of forming a plurality of peptide sequences on a surface of a single substrate comprising the steps of:

a) placing said substrate in contact with a channel block in a first orientation, said channel block having a plurality of channels therein;

b) flowing at least a first amino acid through at least one of said channels, coupling said first to portions of said surface;

c) flowing at least a second amino acid through at least one of said channels, coupling said second amino acid to portions of said surface;

d) rotating said channel block relative to said substrate and placing said substrate in contact with said channel block again;

e) flowing a third amino acid through at least one of said channels to form at least first and second peptide sequences on said surface; and

f) flowing a fourth amino acid through at least one of said channels to form at least third and fourth peptide sequences on said surface.

18. A kit for forming diverse polymer sequences comprising:

a substrate;

a channel block, said channel block having a plurality of grooves therein;

means for holding said channel block in engagement with said substrate;

means for translating said channel block and said substrate relative to the other; and

means for injecting selected reagents into said grooves.

25. The kit as recited in claim 24 wherein said pipettor comprises a plurality of pipettes, each of which is coupled to a different one of said grooves.

26. A system for conducting a plurality of reactions on a single substrate, the system comprising:

at least about 100 reaction regions on the single substrate, each reaction region being capable of conducting a separate reaction;

means for delivering one or more reactants to one or more of the reaction regions; and

means for constraining at least some of the reactants from contacting at least some of the reaction regions.

27. The system recited in claim 26 wherein the substrate comprises a plurality of passages and the reactions are supports within said passages.

28. The system recited in claim 26 wherein the means for delivering the one or more reactants are flow channels of a channel block adjacent the substrate and the means for constraining at least some of the reactants are walls of the flow channel.

29. The system recited in claim 26 wherein the means for constraining at least some of the reactants is a hydrophobic layer on the surface of the substrate.

30. A substrate comprising

greater than about 100 reaction regions having different compounds therein;

a constraining region surrounding the reaction regions, the constraining region being more hydrophobic than the reaction regions.

31. The substrate recited in claim 30, wherein the constraining region comprises hydrophobic protecting groups.

32. The substrate recited in claim 31 wherein the protecting groups are photolabile.

33. The substrate recited in claim 30 wherein the reaction regions define channels.

34. The substrate recited in claim 30 wherein the substrate includes greater than about 1000 reaction regions.

into a second heterogeneous array, wherein the heterogeneous arrays have greater than about 100 distinct reaction regions.

41. The method recited in claim 40 further comprising a step of isolating the first group of reaction regions from the second group of reaction regions.

42. The method recited in claim 41 wherein the first group of reaction regions is isolated by placing a channel block against the substrate.

43. The method recited in claim 40 wherein the first group of reaction regions is isolated from the second group of reaction regions by walls on the substrate.

44. The method recited in claim 43 wherein the substrate includes a series of flow through reaction regions separated from one another by walls.

45. The method recited in claim 40 wherein the first group of reaction regions is isolated from the second group of reaction regions by one or more non-wetting regions on the substrate.

46. The method recited in claim 40 wherein the heterogeneous arrays have greater than about 1000 distinct reaction regions.

47. The method recited in claim 40 further comprising the following steps:

delivering a second reactant to the second group of reaction regions but not to the first group of reaction regions;

allowing the second reactant to react at the second group of reaction regions;

activating a third group of reaction regions, the third group having some reaction regions in common with the first group of reaction regions;

delivering a reactant to the third group or reaction regions but not to the second group of reaction regions; and

allowing the a reaction to take place in the third group of reaction regions.

A A B B
AD D AD D BD D BD
A A B B
AD D AD D BD D BD
A A B B
AC C AC C BC C BC
A A B B
AC C AC C BC C BC

FIG. 3

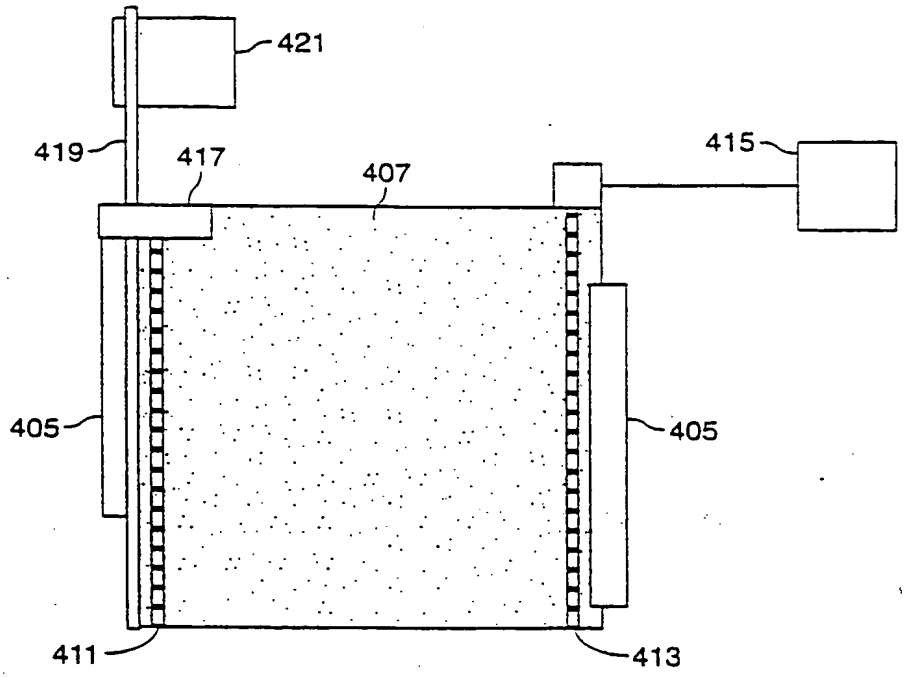
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FIG. 5A

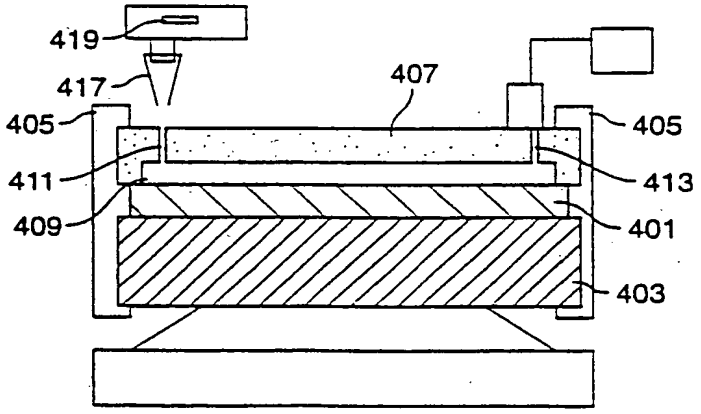
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FIG. 5B

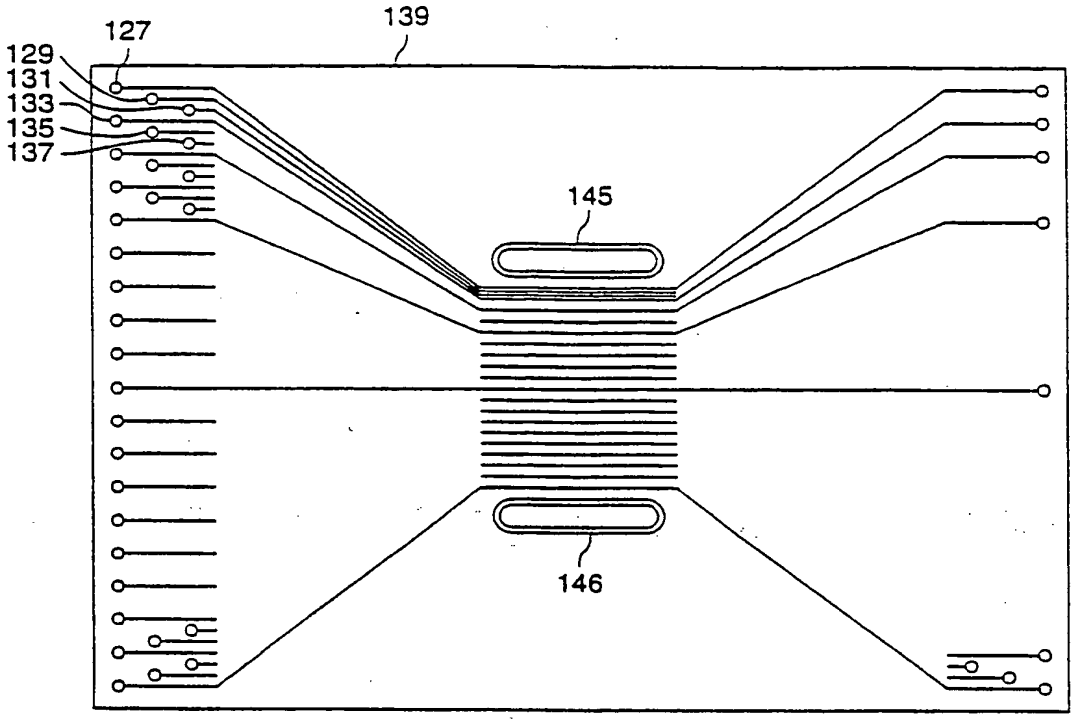

FIG. 7A

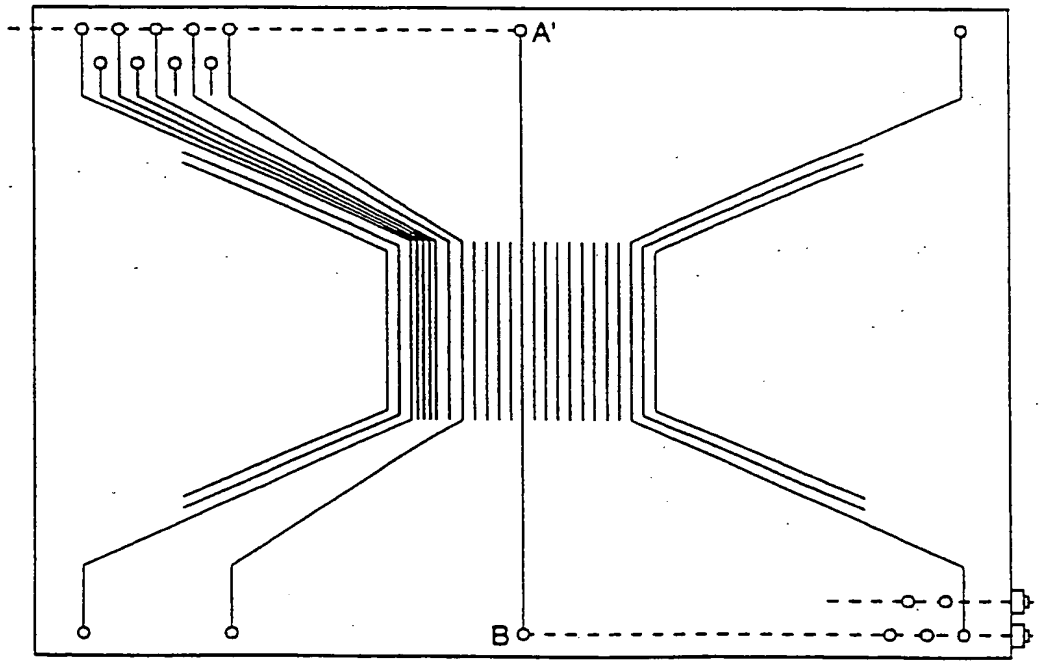

FIG. 7B

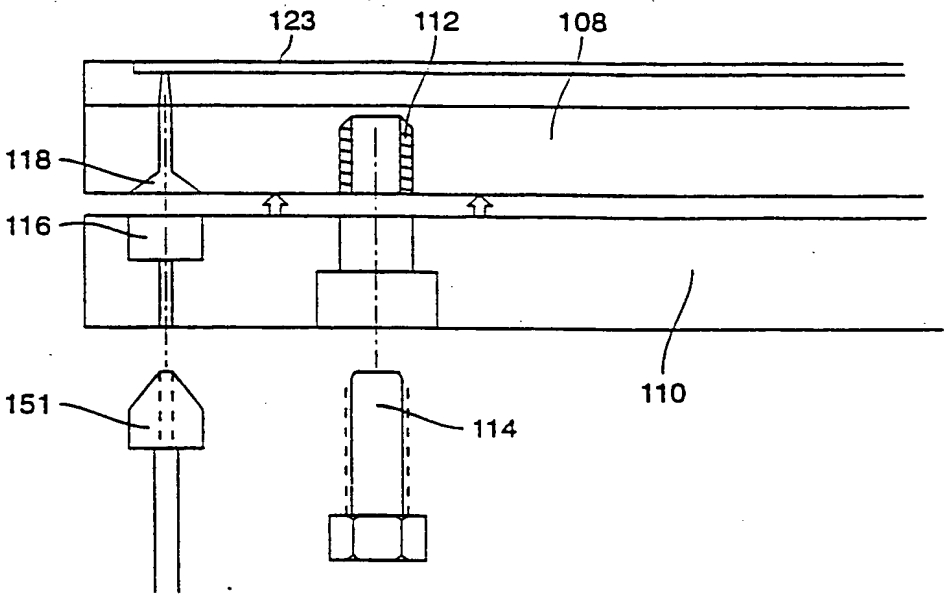

FIG. 9

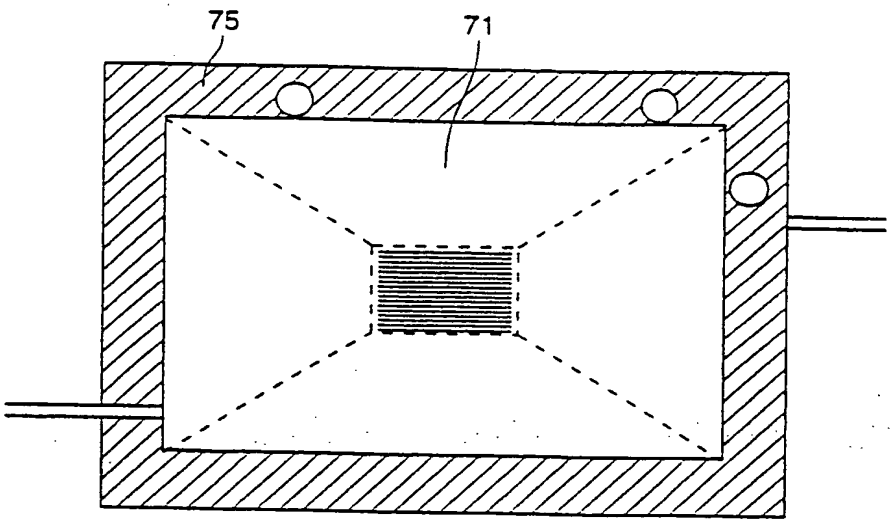

FIG. 11A

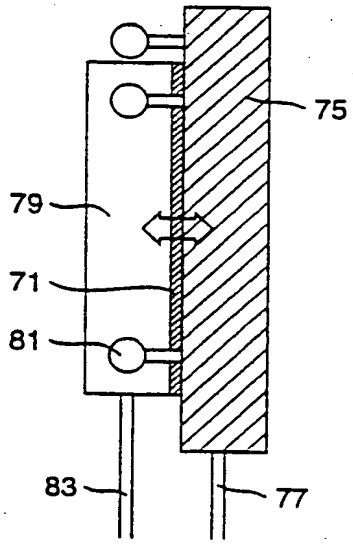

FIG. 11B

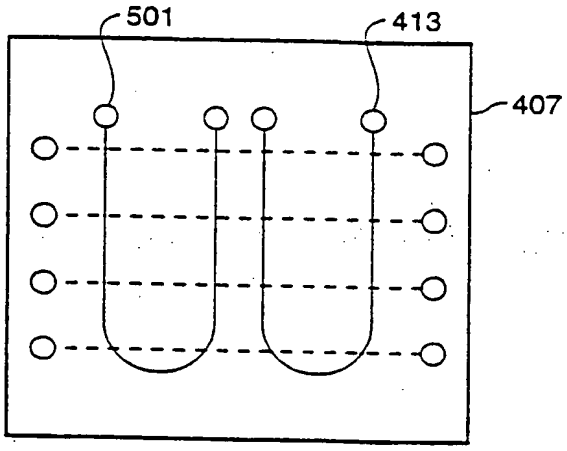

FIG. 13A

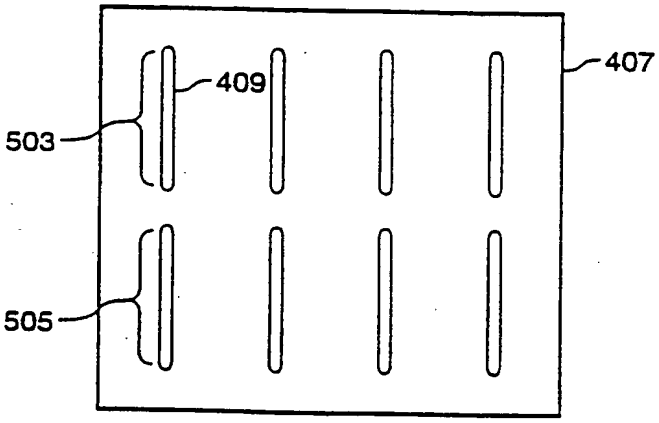

FIG. 13B

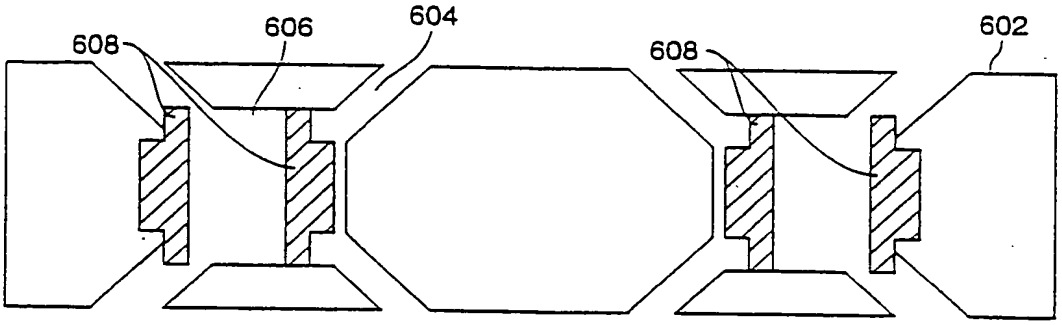

FIG. 15A

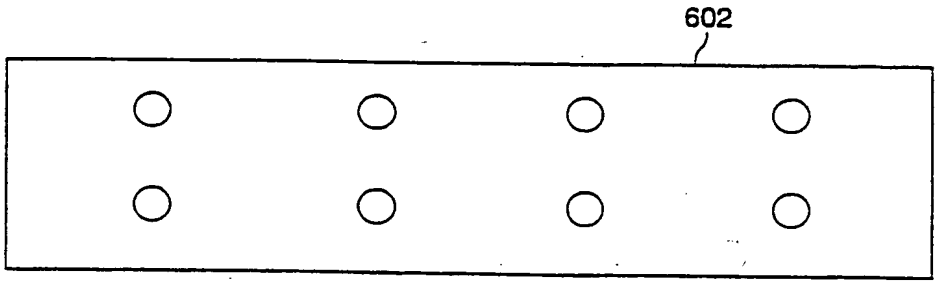

FIG. 15B

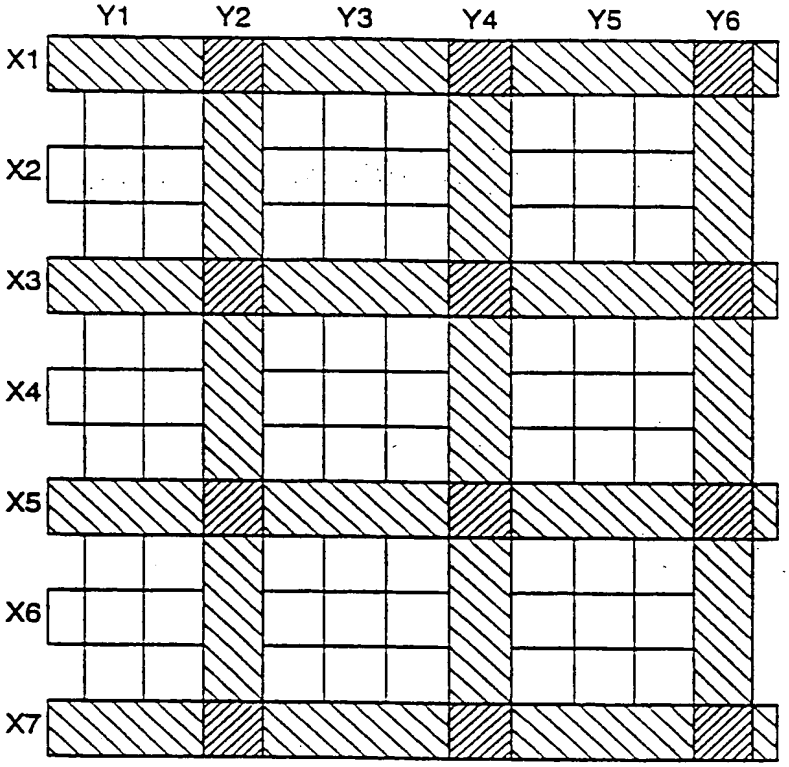

FIG. 17